

## Persistent Borna Disease Virus Infection Confers Instability of HSP70 mRNA in Glial Cells during Heat Stress

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**Borna disease virus (BDV) is a highly neurotropic RNA virus that causes neurological disorders in many vertebrate species. Although BDV readily establishes lasting persistence, persistently infected cells maintain an apparently normal cell phenotype in terms of morphology, viability, and proliferation. In this study, to understand the regulation of stress responses in BDV infection, we investigated the expression of heat shock proteins (HSPs) in glial cells persistently infected with BDV. Interestingly, we found that BDV persistence did not upregulate HSP70 expression even in cells treated with heat stress. Furthermore, BDV-infected glial cells exhibited rapid rounding and detachment from the culture plate under various stressful conditions. Immunofluorescence analysis demonstrated that heat stress rapidly disrupts the cell cytoskeleton only in persistently infected cells, suggesting a lack of thermotolerance. Intriguingly, we found that although persistently infected glial cells expressed HSP70 mRNA after heat stress, its expression rapidly disappeared during the recovery period. These observations indicated that persistent BDV infection may affect the stability of HSP70 mRNA. Finally, we found that the double-stranded RNA-dependent protein kinase (PKR) is expressed at a constant level in persistently infected cells with or without heat shock. Considering the interrelationship between HSP70 and PKR production, our data suggest that BDV infection disturbs the cellular stress responses to abolish antiviral activities and maintain persistence.**

Borna disease virus (BDV) is a neurotropic virus that belongs to the *Mononegavirales* order. Natural BDV infections have been found in a wide variety of vertebrates, suggesting that the host range of this virus probably includes all warm-blooded animals (17, 22). BDV infects the central nervous system (CNS) of many animal species and causes behavioral disturbances reminiscent of autism, schizophrenia, and mood disorders (17, 38, 41, 50). Thus, studies on this virus provide an important paradigm for the mechanisms by which viral infection induces neurobehavioral disorders.

BDV shows noncytopathic replication and long-lasting persistence in both cultured and animal brain cells (10, 51). In immunocompetent rats infected with BDV, a marked immune-mediated meningoencephalitis consistent with classical Borna disease is observed to induce severe neurological disturbances (41, 48). In this model, BDV commonly evades host immune responses after the acute infection phase and establishes life-long persistence, leading to movement disorders (17, 37, 48). On the other hand, neonatal rats infected with BDV develop a tolerant persistent infection without signs of Borna disease or encephalitis (17, 37). Neonatal infection of animals, however, causes neuroanatomical alterations in the developing CNS, especially in the cerebellum and hippocampus, and induces serious neurobehavioral abnormalities (12, 17, 43). These observations have revealed that BDV can directly induce neuronal damage without an immune-mediated mechanism and also

suggested that establishment of a persistent infection in the CNS may be critical for the neuropathogenesis of this virus.

Recent studies have suggested that BDV could modify the microenvironment of infected cells. Hans et al. reported that persistent BDV infection constitutively activated the mitogen-activated protein kinase pathway but efficiently blocked nuclear translocation of activated extracellular signal-regulated kinase (ERK) in PC12 cells (15). Furthermore, we have demonstrated that BDV phosphoprotein (P) specifically interacts with a multifunctional protein, HMGB1 (high-mobility group box 1 protein), and interferes with its functions in persistently infected neural cells (19, 54). More recently, interaction between BDV nucleoprotein (N) and the Cdc2-cyclin B1 complex has been reported to induce decelerated proliferation of infected rat fibroblast cells (36). These findings suggest that although BDV infection appears to be noncytolytic, persistent infection might widely induce functional fragility in infected CNS cells, leading to neurological abnormalities.

Virus infections can induce cellular stress responses, which include the expression of stress response proteins, such as heat shock proteins (HSPs) (21, 44). HSPs mainly work as molecular chaperons and are involved in many biological processes, such as thermotolerance, prevention of misfolding of nascent polypeptides, transmembrane protein transport, nuclear protein transport, and cell viability (24). It has been shown that these stress response proteins are involved not only in cellular maintenance in an infectious environment but also in antiviral action. It has been demonstrated that induction of large HSPs, most notably HSP70, gives rise to antiviral activity during various viral infections, such as influenza virus (35), rhinovirus (8), and human immunodeficiency virus (42). Furthermore, HSPs can induce innate and adaptive immune responses by partici-

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pating in antigen presentation and eliminate virus-infected cells (46). In a mouse model of persistent infection with measles virus, it has been demonstrated that elevated levels of HSP promote cell-mediated viral clearance from the infected CNS (30). On the other hand, many viruses have been shown to use HSPs to stimulate viral entry, transcription, nuclear translocation, and virion formation (3, 7, 14, 26), indicating that HSPs also play roles in the accomplishment of the viral life cycle in targeted host cells. These properties of stress-induced proteins suggest that the regulation of HSPs in infected cells could be critical to the maintenance of viral survival, probably including persistent infection.

This study was thus undertaken to investigate the regulation of stress responses in cells persistently infected with BDV. We demonstrated that persistently infected glial cells are indistinguishable from uninfected parent cells in the expression of large HSPs, as well as in cell morphology and proliferation. Interestingly, we found that heat stress followed by recovery fails to induce HSP70 expression in persistently infected cells. Furthermore, these cells showed rapid rounding and detachment from the culture plate under heat shock stress and oxidative conditions. Immunofluorescence analysis demonstrated that BDV infection easily degrades the actin cytoskeleton and focal adhesion complexes at the lamellipodia with heat shock, supporting the lack of thermotolerance of persistently BDV-infected glial cells. Intriguingly, we found that although infected cells expressed HSP70 mRNA on heating as quickly as did uninfected controls, the mRNA rapidly disappeared during the period of recovery. These results suggested that the persistence of BDV may affect the stability of HSP70 mRNA under stressful conditions. We also demonstrated constant expression of double-stranded RNA-dependent protein kinase (PKR) in infected cells with or without heat shock. Together with the interrelationship between HSP70 and PKR production, we discuss the implications of the regulation of stress responses in cells persistently infected with BDV.

#### MATERIALS AND METHODS

**Cell lines and viruses.** The C6 rat glioma cell line was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. The OL cell line, derived from a human oligodendroglioma, was grown in high-glucose (4.5%) DMEM supplemented with 10% FCS. Cells were passaged every 3 days. Two cell lines were infected with BDV, He/80 (9) and huP2br (29), and after 10 to 30 passages we used these cell lines as persistently infected cells. They were maintained under the same conditions as the parental cell lines. These cells produced infectious BDV.

Primary cortical glial cells were isolated from Lewis rats (SLC, Shizuoka, Japan). Briefly, the brains were gently removed from their skulls and placed in a phosphate-buffered saline (PBS)-filled petri dish on ice. The cortexes were then dissected, dissociated by mechanical trituration, and digested with 2.5% trypsin in PBS plus 5% glucose. DNase I (Sigma-Aldrich, St. Louis, Mo.) was added to a final concentration of 0.01%, and the mixture was incubated for 20 min at 37°C. After the addition of 35% heat-inactivated horse serum, the suspension was centrifuged at 800 × *g* for 3 min. After resuspension in DMEM-Ham's F-12 (1:1) mixed medium, cells were seeded and grown in high-glucose (4.5%) DMEM supplemented with 10% FCS. After 1 to 2 weeks, numerous astrocytes and microglia, as well as oligodendrocytes, were present in cultures, as judged by morphology.

**Stress treatments.** Before experiments, cells (6 × 10<sup>5</sup>) were seeded in 60-mm-diameter culture plates. Semiconfluent cultures were heat shocked at 44°C for 0.5 to 1 h. After the heat shock, the cells were allowed to recover for various times at 37°C. Oxidative stress was administered by incubating the cells with 10 μM hydrogen peroxide for 0.5 h. After being washed with PBS, the cells were

allowed to recover from the treatment for 24 h in normal medium at 37°C. For morphological analysis, cells were visualized under a phase-contrast microscope (Nikon Co., Tokyo, Japan) at various intervals.

**Cell viability assays.** After heat shock, floating cells were carefully collected and quantified. Floating cells were stained with 0.4% trypan blue (Sigma-Aldrich), and numbers of viable and nonviable cells were estimated. For statistical analysis, the data were expressed as the mean plus the standard error. Comparisons of two groups were performed by Student's *t* test with Statcel software (OMS Publishing Inc., Tokyo, Japan).

**Detection of stress-related proteins.** Following stress treatments, the cells were washed with cold PBS and then lysed in ice-cold lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 0.1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 4 mM EDTA, 10 mM NaF, 2 mM Na<sub>2</sub>VO<sub>3</sub>, 2 mM phenylmethylsulfonyl fluoride). After centrifugation (15,000 × *g* for 5 min at 4°C), the soluble fraction was subjected to further analysis. Equal amounts of the proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, Mass.). The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 (TTBS) and then incubated for 2 h with the following primary antibodies: mouse anti-HSP90 (SPA-830; Stressgen Biotechnologies, Inc., San Diego, Calif.), mouse anti-HSP70 (SPA-810; Stressgen), rat anti-HSC70 (SPA-815; Stressgen), mouse anti-HSP60 (SPA-806; Stressgen), mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon International, Temecula, Calif.), rabbit anti-PKR (D-20; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.), rabbit anti-phosphorylated PKR (Calbiochem, La Jolla, Calif.), or rabbit anti-BDV P and N in TTBS at 37°C. After three washes in TTBS, horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, Pa.) were applied for 1 h at 37°C. The membranes were washed three times in TTBS, incubated in commercial enhanced chemiluminescence reagent (ECL Western blotting kit; Amersham Pharmacia Biotech, Uppsala, Sweden), and exposed to X-ray film. The intensity of each reactive band was quantified with NIH Image software.

**Immunofluorescence staining.** Cells were fixed with 4% paraformaldehyde in PBS for 30 min. The fixed cells were permeabilized with PBS containing 0.5% Triton X-100 for 5 min and incubated for 1 h at room temperature in blocking buffer consisting of PBS with 10% FCS. Subsequent antibody incubations were also performed with the blocking buffer. The cells were incubated with anti-focal adhesion kinase (FAK) monoclonal antibody (Transduction Lab, Lexington, Ky.) overnight at 4°C. Subsequently, the cells were washed with PBS three times and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories) for 1 h at room temperature. For detection of filamentous actin (F-actin), rhodamine-phalloidin was mixed with the second antibody solution. After three washes with PBS, the cells were examined with an epifluorescence microscope (Nikon Co.).

**Semiquantitative RT-PCR of HSP70 mRNA.** Total cellular RNA was extracted with TRIzol reagent in accordance with the manufacturer's (Invitrogen, San Diego, Calif.) directions. First-strand cDNAs were synthesized from aliquots of 1 μg of total RNA with the Superscript reverse transcription (RT)-PCR system (GIBCO/BRL, Rockville, Md.). The resulting cDNAs were used as templates for PCR amplification with the primers HSP70 forward (5'-GAG TCC TAC GCC TTC AAT ATG AAG-3') and HSP70 reverse (5'-CAT CAA GAG TCT GTC TCT AGC CAA-3'). PCR was performed with a total volume of 25 μl containing 2 μl of cDNA and 1.25 U of *Taq* polymerase (KlenTaq LA; Sigma-Aldrich). Serial dilutions of positive controls were amplified at 20, 25, 30, and 35 cycles to determine the optimal number of amplification cycles that produces a linear relationship between the input RNA and PCR product. The reaction mixture was preincubated at 94°C for 5 min and then subjected to 30 cycles of PCR at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. As a control for the input RNA, levels of GAPDH (forward primer, 5'-ACC ACA GTC CAT GCC ATC AC-3'; reverse primer, 5'-TCC ACC ACC CTG TTG CTG TA-3') were assayed. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel. Gels were stained with ethidium bromide. Images of agarose gels were captured electronically, and the pixels were inverted. The intensity of each band was quantified with NIH Image software.

**Stability of HSP70 mRNA.** Following heat shock treatment, cells were treated with actinomycin D (10 μg/ml) to globally inhibit transcription, and mRNA levels were subsequently measured in a time course after exposure to the drug for 12 h. Total cellular RNAs were extracted as described above. Aliquots of 20 μg of total RNA were electrophoresed through a 1% agarose gel containing 2.2% formaldehyde and transferred onto nylon membrane (Hybond N+; Amersham) with 20× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate, pH 7). After being baked at 80°C for 2 h, the membrane was hybridized with an [α-<sup>32</sup>P]dCTP-labeled probe specific for rat HSP70 in a buffer containing 5× SSC, 50% form-

amide, 0.1% SDS, 5× Denhardt's solution, and 10 µg of yeast tRNA at 42°C overnight. Rat HSP70-specific probe was synthesized with the 3' end of the rat HSP70 gene as the template by the Random Prime Labeling System in accordance with the manufacturer's (Amersham Pharmacia Biotech) directions. After washing in 0.1× SSC–0.1% SDS at 50°C for 1 h, the specific signals were recorded on X-ray film at –80°C.

## RESULTS

**BDV infection cannot induce HSP expression in glial cell lines.** To understand the regulation of stress responses during BDV persistence, we first investigated the expression of HSPs in two glial cell lines persistently infected with BDV. The C6 and OL cell lines were infected with BDV, and after 10 to 30 passages we used these cell lines (C6/BDV and OL/BDV) as persistently infected cells. Previous studies clearly demonstrated that cell lines persistently infected with BDV show apparently normal morphology and cell viability (22, 51). We confirmed that the persistently infected glial cells were morphologically normal and showed no abnormality in proliferation, although almost 100% of the cells expressed BDV antigens (Fig. 1A and B). To analyze the steady-state levels of HSPs in the cell lines, we performed Western immunoblotting with antibodies against large HSPs, including HSP90, HSP70, HSC70, and HSP60. As shown in Fig. 1C, the persistently infected cells expressed HSPs at levels comparable to those of uninfected cell lines, although we could not detect the expression of HSP70 in uninfected or infected C6 cells. As reported previously (31, 45), large HSPs, such as HSP60, HSC70, and HSP90, were constantly expressed in the cells under normal culture conditions. In addition, acutely BDV-infected cells also showed no differences in HSP expression from the uninfected cells (data not shown). This observation demonstrated that BDV infection cannot upregulate HSP expression in glial cell lines.

**Persistently infected cells fail to express HSP70 during heat stress.** We next investigated whether heat stress followed by recovery can induce the expression of HSPs in persistently infected cells. The cell lines were exposed to a heat stress of 44°C for 1 h with recovery at 37°C, and samples were collected 0, 3, 6, and 12 h after heat treatment. Equivalent amounts of proteins were electrophoresed and reacted with antibodies to HSPs. As shown in Fig. 2, activation of HSP70 gradually occurred and peaked at 12 and 3 h after heat treatment in uninfected C6 and OL cells, respectively. HSP90 expression also appeared to be slightly upregulated in uninfected C6 cells with a peak at 6 h after heat stress (Fig. 2A). Interestingly, only weak expression of HSP70 was detected in persistently infected C6 cells from 6 h after heat treatment (Fig. 2A). On the other hand, no upregulation of HSP70 expression was seen in BDV-infected OL cells during the observation period (Fig. 2B). In addition, activation of BDV N and P expression was not observed in persistently infected cells during the stress response (Fig. 2B). We repeated this experiment at least six times and obtained similar results each time. These results revealed that heat stress induces HSP70 expression less well in glial cells persistently infected with BDV.

**Morphological disruptions of BDV-infected cells by stress treatments.** Previous study has demonstrated that large HSPs, including HSP70, interact with different cytoskeletal components (20). HSP70 appeared to bind mostly to the microtubule

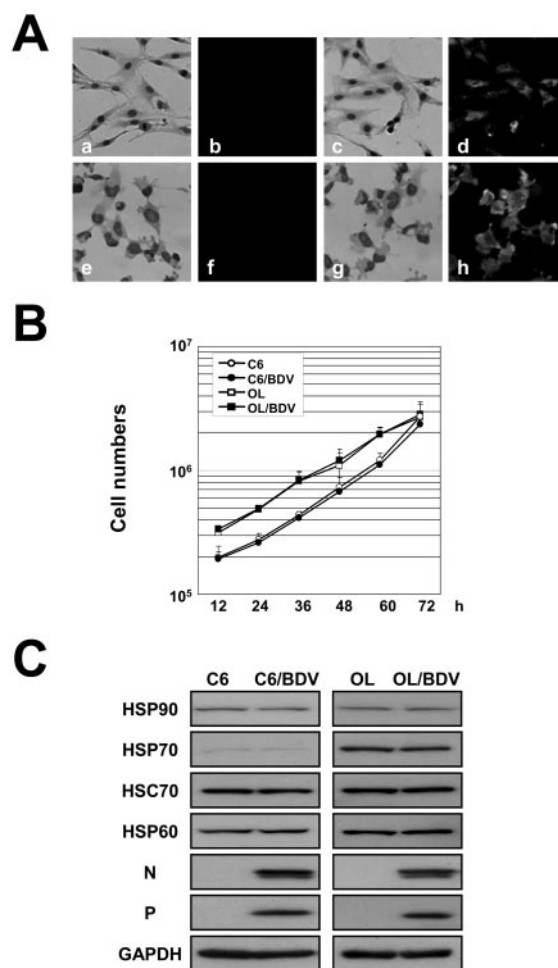


FIG. 1. HSP expression in persistently BDV-infected glial cells. (A) Morphology and BDV antigen expression in persistently BDV-infected glial cell lines. The C6 (a to d) and OL (e to h) cell lines were grown under normal culture conditions. Subpanels: a, b, e, and f, uninfected; c, d, g, and h, persistently infected. The cells were fixed and stained with hematoxylin. BDV antigen was detected by immunofluorescence assay with an anti-P polyclonal antibody. (B) Proliferation of persistently infected glial cells. Cells ( $2 \times 10^5$ ) were seeded in 60-mm-diameter culture plates, and their proliferation was evaluated every 12 h. (C) Expression of large HSPs in persistently infected cell lines. Cells were lysed, and equal amounts of protein were subjected to Western blot analysis with antibodies against HSP90, HSP70, HSC70, HSP60, and BDV P and N. The GAPDH control shows that each lane contains approximately the same amount of protein. Data from one experiment representative of three independent tests are shown.

network and centrosome to play roles in the morphological stabilization of cells during stress responses (20). To understand the effects of a lack of HSP70 induction in cells persistently infected with BDV, therefore, we analyzed the morphological changes in these cells in stressful situations, such as during heat shock and under oxidative conditions. As shown in Fig. 3A, the stressors apparently caused cell rounding in the persistently infected glial cell lines. The rounding was observed within 0.5 h after heat treatment. In contrast, the stressors produced only minimal changes in the morphology of uninfected cells (Fig. 3A). Other types of stress, such as UV treat-



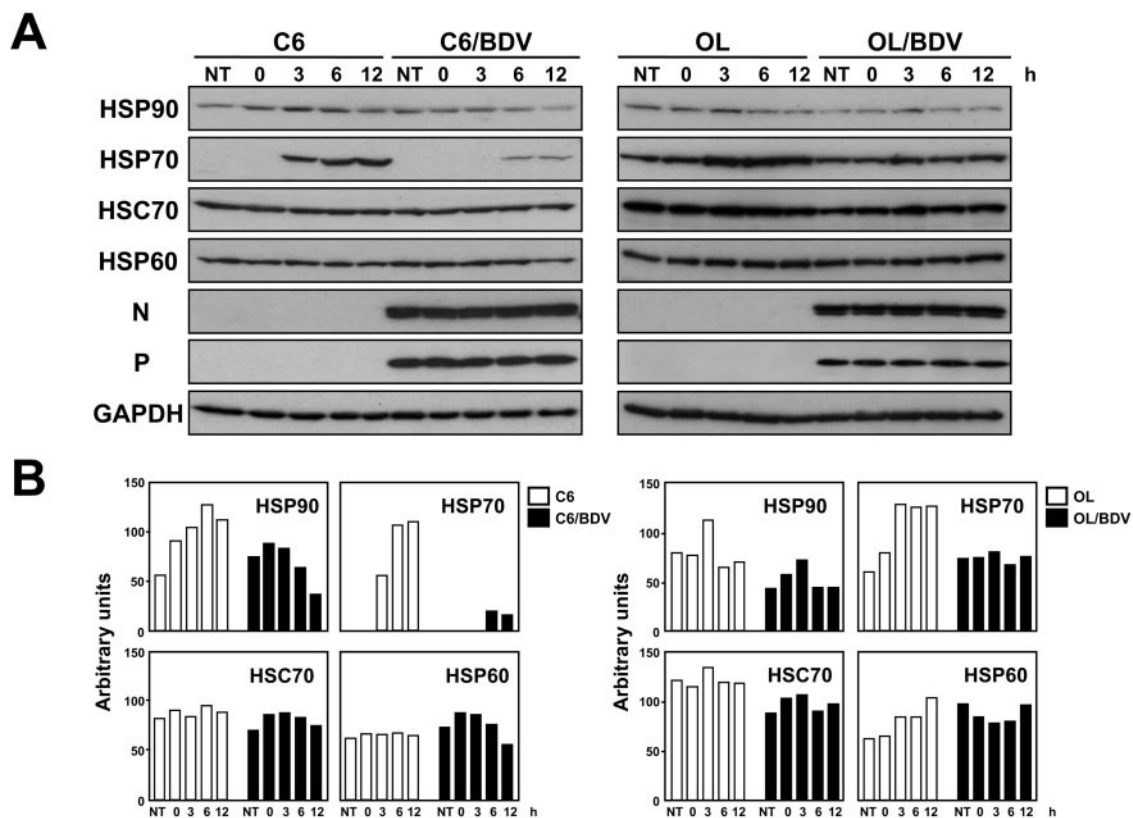


FIG. 2. BDV infection inhibits HSP70 expression induced by heat shock in glial cells. (A) C6 and OL cells were exposed to heat shock at 44°C and allowed to recover at 37°C for different periods. Cells were harvested at the indicated times during the recovery period. Nontreated control cells (NT) were maintained at 37°C and harvested together with heat-stressed cells. After being washed with ice-cold PBS, the cells were lysed and separated by SDS-PAGE (10% acrylamide). Western blot analysis was carried out as described in Materials and Methods. Data from one experiment representative of six independent tests are shown. (B) For quantitative analysis of HSP expression, band intensities were determined with NIH Image software. Values were normalized to GAPDH levels.

ment and serum starvation, also induced cell rounding during the recovery period (data not shown).

Recent studies have shown that HSP70 interacts with FAK, which is a tyrosine kinase, and inhibits the degeneration of FAK to prevent apoptosis (23). FAK is associated with the focal adhesion complex, which is the site of cellular interactions with the extracellular matrix, and plays important roles in integrin-mediated cell adhesion and microtubule stabilization (32). Thus, we next examined whether the rapid rounding and detachment from the culture plate of persistently infected cells resulted from disruptions of the actin cytoskeleton and focal adhesion complex formation in the cells. C6 and C6/BDV cells were exposed to heat stress, and changes in F-actin and focal adhesion complexes were visualized by immunofluorescence staining with rhodamine-phalloidin and anti-FAK antibody, respectively. In uninfected C6 cells, the actin cytoskeleton and focal adhesion complexes were well maintained following heat stress (Fig. 3B and C) whereas heat stress rapidly induced F-actin degeneration in persistently infected C6 cells (Fig. 3B). Furthermore, in C6/BDV cells, the focal adhesion complexes were shown to disappear at the lamellipodia within 1 h after the stress (Fig. 3C). These observations supported a lack of HSP expression in the infected cells and also suggested a sup-

pression of thermotolerance in persistently BDV-infected cells.

Figure 3D shows the total numbers of cells detached from plastic tissue culture dishes by heat treatment. As expected, the floating cell number was significantly higher in persistently infected cells than in uninfected cells. However, interestingly, trypan blue exclusion revealed that more than 85% of the cells floating after heat stress were viable at 1.5 h of recovery in persistently BDV-infected cells (Fig. 3E). In contrast, only 40% of the floating cells remained viable in the uninfected control (Fig. 3E). Upon being replated in a fresh culture dish, infected cells could adhere to the surface within 1 to 2 days and grew normally (data not shown). These results suggested that despite a lack of HSP70 induction, BDV-infected C6 cells are relatively resistant to death from heat shock.

**Altered expression of HSP70 in BDV-infected rat primary glial cells during heat shock.** To understand whether the disturbances found in BDV-infected cultured glial cells is also applicable to an in vivo situation, we also investigated stress responses of rat primary glial cells infected with BDV. At 19 days after infection, primary cells were 75% positive for BDV antigen, with apparently normal morphology and cell viability (Fig. 4A). We exposed primary glial cell cultures to a heat

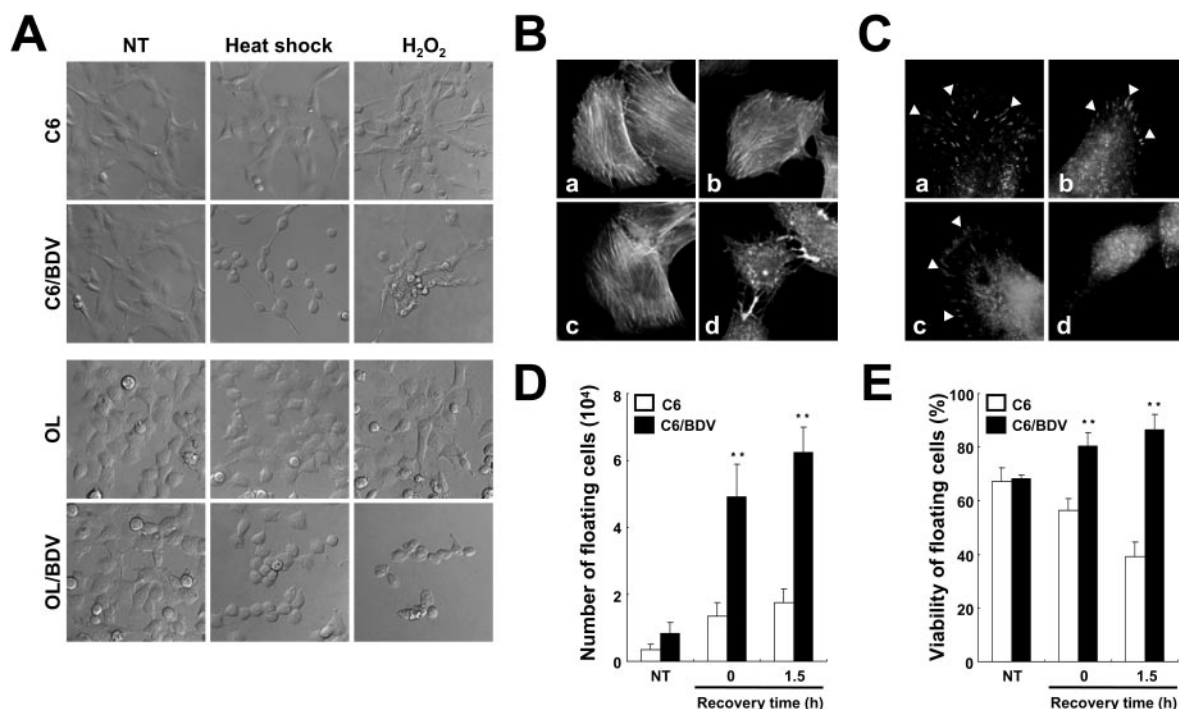


FIG. 3. Suppression of the thermotolerance of persistently infected cell lines. (A) Morphological changes of BDV-infected glial cells. Cells were exposed to heat stress at 44°C for 1 h or to oxidative stress with 10  $\mu$ M hydrogen peroxide for 0.5 h. In the case of oxidative stress, cells were allowed to recover for 24 h in normal medium at 37°C. For morphological analysis, cells were visualized under a phase-contrast microscope. (B and C) Disruption of the actin cytoskeleton and focal adhesion complexes in persistently BDV-infected C6 cells. C6 cells were exposed to heat stress at 44°C for 1 h. Subpanels: a and b, uninfected; c and d, persistently infected. Stress-treated (b and d) and untreated (a and c) cells were fixed and stained with rhodamine-phalloidin (B) or anti-FAK antibody (C). Arrowheads indicate FAK at the lamellipodia. (D) After heat stress, cells were recovered at 37°C. The floating cells in the culture supernatants were counted at 0 and 1.5 h of recovery. (E) The floating cells were stained with 0.4% trypan blue, and the viable cells were counted. The data are expressed as percentages of the total number of cells counted. For statistical analysis, the data were expressed as the mean plus the standard error. Comparisons of two groups were performed by Student's *t* test with the Statcel software. Double asterisks indicate statistically significant differences ( $P < 0.01$ ). NT, nontreated control cells.

stress of 44°C for 1 h and analyzed HSP70 expression, as well as morphological alteration. As shown in Fig. 4B, uninfected primary cells showed significant upregulation of HSP70 from 3 h after heat stress. In contrast, reduced activation of HSP70 was found only from 6 h after treatment in BDV-infected primary glial cells. Furthermore, morphological changes, such as rounding and flatten and shape forms, were observed only in BDV-infected cells within 1 h after treatment (Fig. 4D). This experiment suggested the possibility that altered expression of HSP70 may occur in persistently BDV-infected glial cells even in infected brains.

**BDV causes instability of HSP70 mRNA and PKR activation in persistently infected cells.** Previous studies revealed that expression of heat shock genes is regulated at both the transcriptional and posttranscriptional levels (21, 27). Thus, we next examined the time course of the expression of HSP70 mRNA in C6/BDV cells after heat shock followed by 12 h of recovery at 37°C. As shown in Fig. 5A, HSP70 mRNA expression was rapidly induced by heat shock in both infected and uninfected cells, suggesting that HSP70 mRNA transcription was not affected by BDV persistence. However, interestingly, HSP70 mRNA expression completely disappeared in persistently infected cells by 6 h after heat shock (Fig. 5A). The levels of expression of housekeeping gene and BDV N and P

mRNAs were similar in uninfected and infected C6 cells with or without heat shock, indicating a relatively specific effect of heat on HSP70 mRNA expression. To confirm the instability of HSP70 mRNA in persistently BDV-infected cells, we determined the relative stability of HSP70 mRNA in stress-treated cells. BDV-infected and uninfected C6 cells were heat shocked at 44°C and then allowed to recover at 37°C in the presence of actinomycin D for 12 h. Actinomycin D blocks the de novo synthesis of most mRNAs at the transcriptional level, and therefore kinetic changes in cellular mRNA could reflect the relative degree of turnover. As shown in Fig. 5C, HSP70 mRNA was relatively stable in heat-shocked uninfected C6 cells, and its half-life was estimated as 9 h after heat stress (Fig. 5C). On the other hand, in persistently BDV-infected cells, the level of HSP70 mRNA declined by 50% within the 4-h incubation period (Fig. 5C). These results suggested that BDV persistence is involved in the posttranscriptional stabilization of HSP70 mRNA.

A recent paper has reported that stress-induced PKR stabilizes HSP70 mRNA through interaction with the AU-rich elements present in the 3' untranslated region of the HSP70 mRNA (55). On the other hand, it has also been demonstrated that induction of HSP70 directly plays a role in the suppression of PKR upregulation (25, 33, 34). These studies indicate that

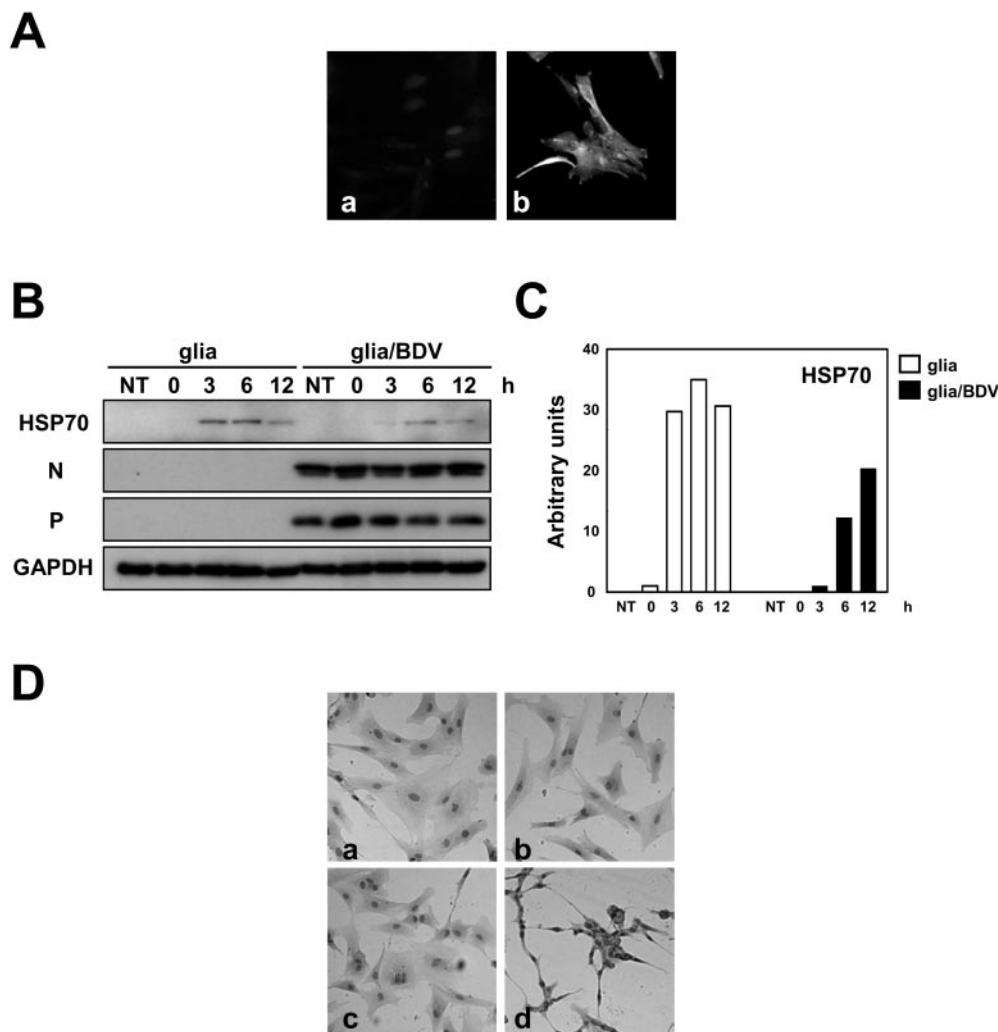


FIG. 4. Effects of BDV infection on HSP70 induction in primary glial cells. (A) Primary glial cells were isolated and infected with BDV as described in Materials and Methods. BDV antigen was detected by immunofluorescence assay with an anti-P polyclonal antibody. Subpanels: a, uninfected; b, BDV infected. (B) Primary glial cells were exposed to heat shock at 44°C and allowed to recover at 37°C for different times. Cells were harvested at the indicated times in the recovery period. Nontreated control cells (NT) were maintained at 37°C and harvested together with heat-stressed cells. After being washed with ice-cold PBS, the cells were lysed and separated by SDS-PAGE (10% acrylamide). Western blot analysis was carried out as described in Materials and Methods. Data from one experiment representative of two independent tests are shown. (C) For quantitative analysis of HSP expression, band intensities were determined with NIH Image software. Values were normalized to GAPDH levels. (D) Morphological changes in BDV-infected primary glial cells. Cells were exposed to heat stress at 44°C for 1 h. For morphological analysis, cells were visualized under a phase-contrast microscope. Subpanels: a and b, uninfected; c and d, persistently infected. Stress-treated (b and d) and untreated (a and c) cells were fixed and stained with hematoxylin.

the interrelationship between HSP70 and PKR plays key roles in the regulation of cellular stress responses. Therefore, we finally investigated the expression of PKR in BDV-infected glial cells. We used an antibody specific for autophosphorylated PKR to estimate its active form in these cells. As shown in Fig. 6A, under normal culture conditions, the active form of PKR appeared to be upregulated in persistently infected cells while uninfected cells showed no production of autophosphorylated PKR. Uninfected C6 cells, however, gradually produced the phosphorylated form of PKR with heat treatment, and the protein drastically increased by 6 to 12 h during the recovery period (Fig. 6B). On the other hand, intriguingly, upregulation of PKR expression was not seen in persistently

infected C6 cells even under stress (Fig. 6). Although our experiment could not exclude the possibility that the level of autophosphorylated PKR had already reached a peak in the infected cells without a stressor, constant expression of PKR along with no HSP70 induction may provide a possible mechanism by which BDV controls cellular stress responses that could oppose persistence.

## DISCUSSION

Our experiments demonstrate that persistent BDV infection may confer instability of HSP70 mRNA in glial cells during the stress response. Although the detailed mechanism by which

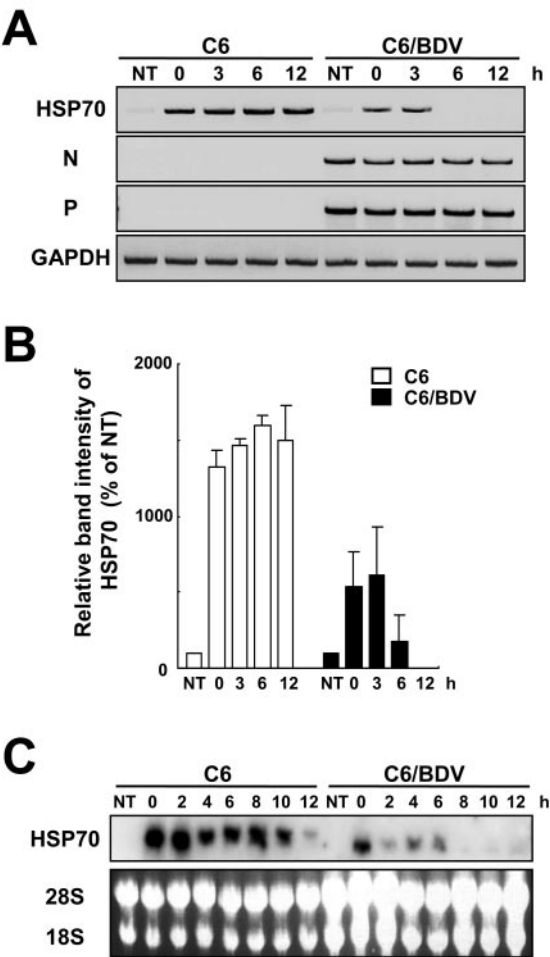


FIG. 5. Stability of HSP70 mRNA in BDV-infected glial cells. C6 cells were exposed to heat stress at 44°C for 1 h and allowed to recover at 37°C for the times indicated. (A) Total RNA was extracted, and semiquantitative RT-PCR for HSP70 mRNA was performed as described in Materials and Methods. As a control for the input RNA, the GAPDH transcript in the cells was also amplified. The amplification products were analyzed by 1.5% agarose gel electrophoresis. Gels were stained with ethidium bromide. The images of agarose gels were captured electronically, and the pixels were inverted. Data from one experiment representative of three independent tests are shown. (B) For quantitative analysis, band intensities were determined with NIH Image software. Values were normalized to GAPDH levels. The data are expressed as the mean plus the standard error. (C) C6 cells were exposed to heat stress at 44°C for 1 h and then allowed to recover at 37°C for various times in the presence of actinomycin D. Total RNA was extracted, and levels of HSP70 mRNA in the extract were analyzed by Northern blotting as described in Materials and Methods. The levels of 28S and 18S rRNAs are also shown. NT, nontreated control cells.

BDV affects the posttranscriptional step of the HSP70 mRNA has remained unclear, the interrelationship between the functions of HSP70 and PKR provides a possibility that aberrant activation of PKR contributes to the instability of the mRNA in BDV-infected cells. PKR is a serine/threonine kinase that is activated by not only RNA or DNA viral infections but also heat stress and several chemical stressors. The best-characterized role of this kinase is the downregulation of mRNA trans-

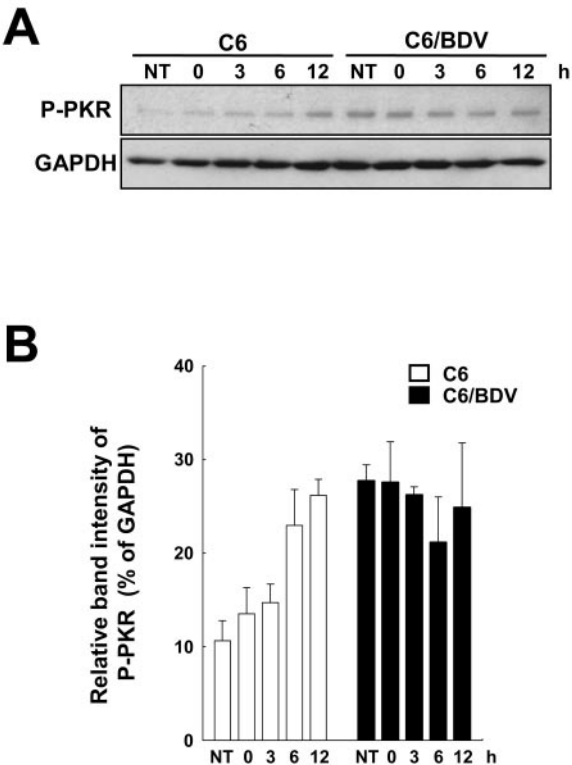


FIG. 6. Induction of autophosphorylated PKR in BDV-infected glial cells during heat stress. (A) After heat shock, uninfected and BDV-infected C6 cells were washed with ice-cold PBS and lysed in lysis buffer. Nontreated control cells (NT) were maintained at 37°C and harvested together with heat-stressed cells. Equivalent amounts of proteins were separated by SDS-PAGE (10% acrylamide). Western blot analysis was carried out to examine relative levels of phosphorylated PKR (P-PKR). Data from one experiment representative of three independent tests are shown. (B) For quantitative analysis, band intensities were determined with NIH Image software. Values were normalized to GAPDH levels. The data are expressed as the mean plus the standard error.

lation via phosphorylation of the  $\alpha$  subunit of eukaryotic initiation factor 2, and activation of PKR results in cell growth arrest and apoptosis, as well as increased antiviral interferon activity (5, 52). We propose that PKR function is disrupted in cells persistently infected with BDV, for the following reasons. First, although PKR expression is upregulated in persistently BDV-infected cells in a steady state, the cells did not exhibit any disturbances in proliferation, viability, or BDV protein synthesis (Fig. 1). Second, the constant activation of PKR did not induce HSP70 upregulation in infected cells (Fig. 1 and 2). Third, persistently infected glial cells appeared not to undergo cell death even during the heat shock response period (Fig. 3). In uninfected cells, on the other hand, PKR activation by heat shock seems to be linked to the induction of HSP70 expression and cell death (Fig. 2 and 3). In addition, PKR activation without inhibition of viral protein synthesis was also demonstrated in BDV-infected rat brains (11). Moreover, it has been reported that interferon induction is too weak to eliminate viral activities in BDV-infected mouse brains (47). From these observations, it is likeliest that BDV inhibits PKR function. Recent studies revealed that many viruses can disturb PKR in



infected cells in order to avoid its antiviral actions and cell apoptosis. It is evident that herpes simplex virus infection downregulates eukaryotic initiation factor 2 $\alpha$  phosphorylation although PKR is activated (6, 13, 16). Furthermore, simian virus 40 appears to reverse PKR-mediated translational inhibition at a step downstream of PKR activation (13, 39). Thus, one might argue that persistent BDV infection also inhibits the PKR signaling pathway somewhere downstream of PKR autophosphorylation. Note that we found some discrepancy between the expression kinetics of HSP70 protein and mRNA in BDV-infected cells (Fig. 2 and 5). We found weak activation of HSP70 protein in persistently infected C6 cells at 6 and 12 h after heat stress, whereas HSP70 mRNA was detected at 0 and 3 h by RT-PCR. Interestingly, a previous study has also demonstrated that slight activation of HSP70 is detected later than expression of its mRNA for 4 h in a PKR-negative cell line (55). On the basis of these observations, it is possible that inhibition of the PKR pathway may also affect the delayed accumulation of HSP70 in stress-treated cells.

Inhibition of HSP70 induction might be critical for the survival of the virus in host cells. HSP70 is known to play direct roles in interfering with viral protein synthesis and replication (8, 35, 42). In addition, this protein can facilitate viral antigen presentation in cells such as macrophages and dendrites (46, 49). Furthermore, elevations of intracellular HSP levels have been shown to improve cell tolerance to inflammatory cytokines, such as tumor necrosis factor  $\alpha$  and interleukin-1 (1). Moreover, the HSP70 present on the cell surface functions as a recognition molecule for natural killer cells (28). These observations demonstrated that HSP70 plays important roles in viral elimination in infected host cells. Thus, suppression of HSP70 expression could be an effective strategy used by BDV to maintain long-lasting persistence.

In the CNS, a number of HSPs are constitutively or inducibly expressed, and their upregulation and presence are connected to the neuroprotection of neuronal and glial cells (40, 53). It is generally accepted that HSP70 is highly upregulated by hyperthermia and a variety of other stressors in astrocytes, oligodendrocytes, and microglia, and the released HSP70 can enhance neuronal stress tolerance (40, 53). Another study has also demonstrated that HSP70 causes microglial activation and an increase in cytokine production, which might contribute to neuroprotective roles in infected brains (18). Furthermore, large HSPs are known to interact with the cytoskeleton and assist in the proper assembly and spatial organization of cells (20). In neuronal cells, cytoskeletal stability could be absolutely necessary for synaptic plasticity and CNS development. Indeed, it has been reported that HSP70 localizes to the synapses after stress induction and also that the expression of HSP70 protects synapse formation, as well as synaptic transmission (2, 31, 40). Interestingly, we also found that a human neuroblastoma cell line, SK-N-SH, shows delayed expression of HSP70 during persistent BDV infection. Expression of HSP70 was detected only from 12 h after heat shock in infected SK-N-SH cells, while uninfected control cells rapidly induced HSP70 from 3 h after heat treatment (data not shown). These observations suggest that inhibition of HSP70 expression during BDV infection may have deleterious effects on synaptic plasticity, especially under stress. Moreover, HSP70 has been revealed to be constitutively expressed in the rat CNS from

postnatal development to maturity (4), suggesting that inhibition of HSP70 expression in developing brains may cause developmental damage of the CNS, as is shown in the brains of rat neonatally infected with BDV. Further study is needed to understand the effects of HSP70 inhibition in the brains of animals persistently infected with BDV.

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